

1 **COMPOSITIONS AND METHODS FOR INHIBITING ANGIOGENESIS**

2
3 **CROSS-REFERENCE TO RELATED APPLICATIONS**

4 The present nonprovisional patent application is a Continuation-In-Part of
5 Application No. 09/935,145 filed August 22, 2001, which in turn claims benefit of
6 provisional patent application entitled "Composition and Methods for Inhibiting
7 Angiogenesis" with filing date August 22, 2000 and patent application number
8 60/227,152; each of which is herein incorporated by reference.

9
10 **STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR**
11 **DEVELOPMENT**

12 The United States government may have certain rights in the present invention
13 pursuant to grant number SBIR/1R43CA094698-01 from the National Institutes of
14 Health.

15
16 **BACKGROUND OF THE INVENTION**

17 Angiogenesis or neovascularization is the formation of new blood vessels from
18 pre-existing capillaries via a mechanism that involves degradation of the basement
19 membrane which surrounds the parent vessel, migration of endothelial cells through the
20 degraded membrane, proliferation of the migrating cells, endothelial cell differentiation,
21 and loop formation (Folkman, J., Angiogenesis and angiogenesis inhibition: an overview,
22 *EXS.*, **79**, 1-8 (1997)). With the exception of wound healing and menstruation,
23 angiogenesis in adults is observed only in pathological situations such as cancer,

1 atherosclerosis, and psoriasis, where it contributes to the progression and symptom
2 manifestation of the disease (Folkman, J. Angiogenesis in cancer, vascular, rheumatoid
3 and other disease, *Nat. Med.* **1**(1), 27-31 (1995)). Other “angiogenesis-related” diseases
4 include endometriosis, Kaposi’s sarcoma and other HIV-related conditions, leukemia,
5 scleroderma, pyogenic granuloma, myocardial angiogenesis, corneal diseases, rubeosis,
6 neovascular glaucoma, diabetic retinopathy, macular degeneration, and retrolental
7 fibroplasia. As used herein, the term “angiogenesis-related diseases” means pathological
8 conditions that require endothelial cell proliferation for progression and symptom
9 manifestation (Chappey, O., *et al.* Endothelial cells in culture: an experimental model for
10 the study of vascular dysfunctions. *Cell Biol. Toxicol.*, **12**(4-6), 199-205 (1996)).

11 Increasing experimental evidence suggest that angiogenesis plays an essential role
12 in cancer development. It has been observed that solid tumors neither grow beyond 1-2
13 mm³ nor metastasize unless they become vascularized (Folkman, J. What is the Evidence
14 that Tumors are Angiogenesis Dependent?, *J. Natl. Canc. Inst.*, **82**, 4-6 (1990)).
15 Formation of tumor vasculature is necessary in order to deliver nutrients and oxygen at
16 the tumor site, thus, providing a route for tumor metastasis to distant sites. Compositions
17 that inhibit endothelial cell proliferation and/or migration have been shown to inhibit
18 tumor neovascularization, and to prevent tumor growth and metastasis (Eatock, M.M., *et*
19 *al.* Tumour vasculature as a target for anticancer therapy. *Cancer Treat Rev.* **26**(3), 191-
20 204 (2000)). Several of these inhibitors are currently under evaluation in human clinical
21 trials (Deplanque, G., *et al.* Anti-angiogenic agents: clinical trial design and therapies in
22 development, *Eur. J. Cancer*, **36**, 1713-1724 (2000)).

1 Antibodies are proteins synthesized by B lymphocytes usually in response to the
2 presence of a foreign substance, called an antigen (Askonas, B.A. Immunoglobulin
3 synthesis and its induction in B-lymphoid cells, *Acta Endocrinol Suppl (Copenh)*, **194**,
4 117-132 (1975)). Antibodies are the recognition elements of the humoral immune
5 response, designed to lyse foreign microorganisms and infected cells via activation of the
6 complement system. Antibodies possess specific affinity for the antigens that induced
7 their formation and they readily complex with them to trigger complement activation.
8 Naturally occurring antibodies consist of two heavy and two light chains linked together
9 by disulfide bonds. Each chain comprises domains of unique sequence responsible for
10 antigen binding (variable domains) and domains of constant sequence involved in
11 complement activation and mediation of antibody-dependent cellular toxicity (constant
12 domains). Furthermore, the variable domains of light (V_L) and heavy (V_H) chains have
13 similar structure with each domain comprising four somewhat conserved regions, called
14 the framework regions (FR), and three hyper-variable regions, called complementarity
15 determining regions (CDR). Studies have shown that CDRs determine antibody
16 specificity (Ohno *et al.* Antigen-binding specificities of antibodies are primarily
17 determined by seven residues of V_H , *Proc Natl Acad Sci U S A*, **82(9)**, 2945-2949
18 (1985)). In V_H chains, CDRs are located in the proximity of positions 30-35 (CDR1), 50-
19 65 (CDR2), and 95-102 (CDR3) (Kabat *et al.* *Sequences of Proteins of Immunological*
20 *Interest*, 5th edit., NIH Publication no 91-3242 US. Department of Health and Human
21 Services (1991) and Honegger *et al.*). Yet another numbering scheme for
22 immunoglobulin variable domains: an automatic modeling and analysis tool. *J. Mol. Biol.*

1 309, 657-670 (2001)). In V_L chains, CDRs are located in the proximity of positions 24-
2 34 (CDR1), 50-56 (CDR2), and 89-97 (CDR3).

3 Antibodies produced in response to the presence of a single antigen have a
4 common specificity but they are heterogeneous in nature, since they are derived from
5 many different antibody-producing cells. Homogeneous or monoclonal antibodies can be
6 produced through hybridoma cells (Galfre, G. and Milstein, C. Preparation of
7 monoclonal antibodies: strategies and procedures, *Methods Enzymol.*, **73(Pt B)**, 3-46
8 (1981)). The hybridoma cell method of producing large amounts of homogeneous
9 populations of antibodies with a particular specificity has allowed the use of monoclonal
10 antibodies as diagnostic and therapeutic agents (Milstein, C. With the benefit of
11 hindsight, *Immunol. Today*, **21(8)**, 359-64 (2000)).

12 Initially, animal-derived monoclonal antibodies had limited therapeutic value in
13 humans due to antigenicity. The problem was solved with the production of humanized
14 antibodies. Humanized antibodies are defined as immunoglobulin variants or fragments
15 capable of binding to a predetermined antigen and which comprise a FR region having
16 substantially the amino acid sequence of a human immunoglobulin and a CDR region
17 having substantially the amino acid sequence of a non-human immunoglobulin (Hurle,
18 M.R. and Gross, M. Protein engineering techniques for antibody humanization, *Curr.*
19 *Opin. Biotechnol.*, **5(4)**, 428-33(1994)). Humanized antibodies have been recently
20 approved for the treatment of various diseases including cancer. Trastuzumab, a
21 humanized antibody against HER-2 receptor, is used for the treatment of breast cancer,
22 while Rituximab, a humanized antibody against CD20, is used for the treatment of
23 lymphoma (Baselga, et al. Phase II study of weekly intravenous trastuzumab (Herceptin)

1 in patients with HER2/neu-overexpressing metastatic breast cancer, *Semin. Oncol.*, **26**(4
2 **Suppl 12**), 78-83 (1999); Slamon et al. Use of chemotherapy plus a monoclonal
3 antibody against HER2 for metastatic breast cancer that overexpresses HER2, *N. Engl. J.*
4 *Med.*, **344**(11), 783-92 (2001); Byrd et al. Rituximab using a thrice weekly dosing
5 schedule in B-cell chronic lymphocytic leukemia and small lymphocytic lymphoma
6 demonstrates clinical activity and acceptable toxicity, *J. Clin. Oncol.*, **19**(8), 2153-64
7 (2001)).

8 Humanized antibodies are usually produced recombinantly. Recombinant
9 production of immunoglobulin variants or fragments requires: a. the isolation and
10 sequencing of the DNA encoding the immunoglobulin variants or fragments and b. the
11 insertion of the isolated DNA into a replicable vector for further cloning (amplification)
12 or expression. DNA encoding fragments of interest is often isolated from cDNA by
13 using appropriate oligonucleotide probes capable of binding to specific genes (e.g., those
14 encoding the heavy and light chains of the monoclonal antibodies). cDNA is obtained by
15 reverse transcription of RNA isolated via conventional methods from hybridoma cells
16 producing the monoclonal antibody of interest. Once isolated, DNA may be placed into a
17 variety of expression vectors, which are then transfected into host cells such as *E. coli* or
18 Chinese hamster ovary (CHO) cells for intracellular or extracellular production of the
19 antibody variant or fragment of interest. Intracellular production of an antibody or
20 antibody fragment requires its purification from lysates of host cells, while extracellular
21 production requires purification from supernatants of host cells.

22 Typically, recombinant antibody constructs consist of modified forms of the
23 antigen-binding portion of an antibody, also known as Fv. Single chain Fv molecules

(scFv) usually comprise V_H and V_L domains joined with a small peptide linker in a single polypeptide chain. scFv molecules exhibiting antitumor properties are particularly desired in cancer therapy because their small size (~30 Kd) allows for tumor penetration. Unfortunately, their small size also facilitates increased blood clearance (Hudson *et al.* Recombinant antibody constructs in cancer therapy, *Curr. Opin. Immunol.*, **11**, 548-557 (1999)). To reduce blood clearance and to increase functional affinity (scFv are monovalent), scFv dimers and trimers have been produced. Formation of multimeric scFv complexes usually depends on the length of the linker between V_H and V_L domains. Short linkers (5-10 residues) result in the formation of scFv dimers (also known as diabodies), while linkers with less than three residues in length result in the formation of trimers (also known as triabodies) (Holliger *et al.* "Diabodies": Small bivalent and bispecific antibody fragments, *Proc. Natl. Acad. Sci. USA*, **90**, 6444-6448 (1993) and Atwell *et al.* ScFv multimers: length of the linker between V_H and V_L domain dictates precisely the transition between diabodies and triabodies, *Protein Eng.*, **12**, 597-604 (1999)).

BRIEF SUMMARY OF THE INVENTION

In accordance with the present invention, compositions and methods are provided for inhibiting angiogenesis and for treating angiogenesis-related diseases.

The compositions provided herein comprise naturally occurring or synthetic peptides containing an amino acid sequence of the following motif :



1 where $a=2-3$, $b=2-3$, $c=3-4$, J is a positively charged amino acid, i.e., arginine (R) or
 2 lysine (K), Z is a negatively charged amino acid, i.e., aspartic acid (D) or glutamic acid
 3 (E), and X is any amino acid.

4 Examples of naturally occurring or synthetic peptides containing the amino acid
 5 sequence of JJZX_aZ_bJX_cJXJXJZ include the following:

6 F G K R E Q A E E E R Y F R A Q S R E Q L A A L (SEQ ID NO: 1)

7 F G K R E Q A E E E R Y F R A R A K E Q L A A L (SEQ ID NO: 2)

8 F V K R E R A T E D F F V R Q R E K E Q L R H L (SEQ ID NO: 3)

9 An example of a naturally occurring or synthetic peptide containing the amino
 10 acid sequence of ZZZJXXXXJXJJXXJ includes the following:

11 G M D E L S E E D K L T V S R A R K I Q R F (SEQ ID NO: 4)

12 In further embodiments, the invention provides compositions comprising
 13 antibodies that bind to peptides containing an amino-acid sequence of the previously
 14 mentioned motif (1). In a yet another embodiment, the invention provides compositions
 15 comprising scFv molecules of an antibody binding to SEQ ID NO: 4.

16 The methods provided herein for treating angiogenesis-related diseases involve
 17 administering to a human or animal a composition containing therapeutic dosages of a
 18 naturally occurring protein, protein fragments, or peptides containing an amino acid
 19 sequence of the previously mentioned motif (1).

20 In further embodiments, the invention provides methods for treating angiogenesis-
 21 related diseases comprise administering to a human or animal a composition containing
 22 therapeutic dosages of an antibody that binds to a peptide containing an amino acid
 23 sequence of the previously mentioned motif (1).

1 Thus, it is an object of the present invention to provide compositions and methods
2 for inhibiting angiogenesis.

3 It is another object of the present invention to provide methods and compositions
4 for treating cancer by inhibiting tumor neovascularization.

5 6 BRIEF DESCRIPTION OF DRAWINGS

7 FIG. 1 is a schematic diagram showing the sequence of peptides EP01 (SEQ ID
8 NO: 1), EP02 (SEQ ID NO: 4), and EP03 (SEQ ID NO: 7) containing amino acid
9 sequences of the previously mentioned motif (1).

10 FIG 2. is a graph depicting the ability of peptides EP01 (SEQ ID NO: 1) (open
11 squares), EP02 (SEQ ID NO: 4) (open diamonds), and EP03 (SEQ ID NO: 7) (open
12 circles) to inhibit basic fibroblast growth factor (bFGF)-induced proliferation of human
13 umbilical vein endothelial cells (HUVECs).

14 FIG. 3A and FIG. 3B are graphs depicting the ability of murine polyclonal
15 antisera raised against peptides EP02 (SEQ ID NO: 4), designated herein as anti-EP02,
16 and EP03 (SEQ ID NO: 7), designated herein as anti-EP03, and normal murine serum to
17 specifically bind to peptides EP02 (SEQ ID NO: 4) (FIG. 3A) and EP03 (SEQ ID NO: 7)
18 (FIG. 3B).

19 FIG. 4 is a graph depicting the ability of a murine anti-EP02 monoclonal antibody
20 (mab), named B2G4, and a murine anti-EP03 mab, named D2G11, to specifically bind on
21 the cell surface of HUVECs.

22 FIG. 5 is a graph depicting the ability of B2G4 and D2G11 to inhibit bFGF-
23 induced proliferation of HUVECs.

FIG. 6 is a diagram depicting the DNA sequence (A) encoding the V_H domain of B2G4 and the corresponding amino acid sequence (B). Highlighted amino acid sequences indicate potential CDRs.

FIG. 7 is a diagram depicting the DNA sequence (A) encoding the V_L domain of B2G4 and the corresponding amino acid sequence (B). Highlighted amino acid sequences indicate potential CDRs.

Compounds according to the invention

As described below, compounds, which are useful in accordance with the invention, include naturally occurring and synthetic peptides containing an amino acid sequence of the previously mentioned motif (1) and antibodies that bind to naturally occurring and synthetic peptides containing an amino acid sequence of the previously mentioned motif (1). Synthetic peptides include but are not limited to peptides EP01 (SEQ ID NO: 1) and EP02 (SEQ ID NO: 4). Naturally occurring peptides include but are not limited to F₁-ATPase inhibitor protein (F₁I) (SEQ ID NO: 5) and the beta (β) subunit of F₁-ATPase (SEQ ID NO: 6).

DETAILED DESCRIPTION OF THE INVENTION

Other objects, features and aspects of the present invention are disclosed in, or are obvious from, the following Detailed Description. It is to be understood by one of ordinary skill in the art that the present discussion is a description of exemplary embodiments only and is not intended as limiting the broader aspects of the present invention, which broader aspects are embodied in the exemplary construction.

1 The present invention comprised of methods and compositions for treating
2 angiogenesis-related diseases in a human or animal. The treatment comprises the
3 administration of a peptide or antibody in sufficient amount to inhibit endothelial cell
4 proliferation or migration and to suppress angiogenesis-related diseases.

5 *I. Definitions*

6 The terms “a”, “an” and “the” as used herein are defined to mean one or more and
7 include the plural unless the context is inappropriate.

8 The term “peptides” relates to chains of amino acids whose alpha carbons are
9 linked through peptide bonds formed by a condensation reaction between the carboxyl
10 group of the alpha carbon of one amino acid and the amino group of the alpha carbon of
11 another amino acid. A peptide has two terminal amino acids, one amino acid with a free
12 amino-group called the amino- or N-terminus and one amino acid with a free carboxyl
13 group called the carboxyl- or C-terminus. In a peptide, amino acids are numbered
14 starting at the amino terminus and increasing in the direction of the carboxyl-terminus.

15 Peptides are produced chemically or recombinant. Solid phase is the preferred
16 method for chemical synthesis of peptides. It involves the attachment of the C-terminal
17 amino acid to an insoluble support and the sequential addition of the remaining amino
18 acids. An alternative method for synthesizing amino acids is the recombinant nucleic
19 acid method, which involves the generation of a nucleic acid sequence encoding the
20 peptide, followed by the expression of the peptide in a host and isolation and purification
21 of the expressed peptide.

22 The term “antibody” refers to monoclonal, polyclonal, multispecific (formed from
23 at least two intact antibodies), or humanized antibodies as well as antibody fragments so

1 long as they possess the desired biological activity. Monoclonal antibodies are obtained
2 through the hybridoma method or the recombinant DNA method, or isolated from phage
3 display antibody libraries. Techniques for antibody production through the previously
4 mentioned methodologies are known to those skilled in the art. Multispecific or chimeric
5 antibodies are prepared using synthetic proteins methods known in the art. Humanization
6 of an antibody can be achieved by substituting non-human CDRs for the corresponding
7 sequences of a human antibody as described by Jones et al., *Nature*, 321: 522-525 (1986)
8 and Riechmann et al., *Nature*, 332:323-327 (1988). Antibody fragments can be produced
9 via proteolytic digestion or recombinant methods known in the art.

10 As used herein, the term “single chain Fv or scFv” molecule refers to a
11 recombinantly produced antibody fragment comprising the V_H and V_L domains of an
12 antibody in a single polypeptide chain. Usually, an scFv molecule also includes a short
13 amino acid sequence between the V_H and V_L domains, which enables the scFv molecule
14 to form the appropriate structure for antigen binding. As used herein, the term “linker”
15 refers to the amino acid sequence that links V_H to V_L in an scFv molecule.

16 As used herein, the terms “diabody” and “triabody” refer to complexes consisting
17 of two and three scFv molecules, respectively.

18 As used herein, the term “angiogenesis-related” diseases refers to pathological
19 situations that require formation of new blood vessels for progression and symptom
20 manifestation. Such diseases include, but are not limited to, cancer (solid tumor and
21 leukemias), granulomas, abnormal wound healing, atherosclerosis, rheumatoid arthritis,
22 psoriasis, diabetic retinopathy, macular degeneration, endometriosis, and Kaposi’s
23 sarcoma, diabetic neovascularization, peptic ulcer, and scleroderma.

1 Antibodies and antibody-binding fragments with sequences homologous to those
2 described herein are also included in the present invention. Homologues are those
3 antibodies and antibody-binding fragments with amino acid sequences that have sequence
4 identity or homology with amino acid sequence of the B2G4 antibodies of the present
5 invention. Preferably identity is with the amino acid sequence of the variable regions of
6 the B2G4 antibodies of the present invention. "Sequence identity" and "sequence
7 homology" as applied to an amino acid sequence herein is defined as a sequence with at
8 least about 90%, 91%, 92%, 93%, or 94% sequence identity, and more preferably at least
9 about 95%, 96%, 97%, 98%, or 99% sequence identity to another amino acid sequence,
10 as determined, for example, by the FASTA search method in accordance with Pearson
11 and Lipman, Proc. Natl. Acad. Sci. USA 85, 2444-2448 (1988).

12
13 II. *Suitable Methods for Practicing the Invention*

14 Inhibition of Endothelial Cell Proliferation

15 Anti-angiogenic activity is evaluated by testing the ability of a peptide or an
16 antibody to inhibit endothelial cell growth *in vitro*. An endothelial cell proliferation
17 assay typically involves the routine culturing of the endothelial cells to confluency in the
18 appropriate media. Subsequently, the cells are trypsinized and plated in a 96-well plate at
19 5,000 cell per well. The cells are cultured for 96 hours in the presence of the peptide or
20 antibody and growth factors. Cell proliferation is then determined using
21 spectrophotometry (MTT assay, BrdU assay) or fluorimetry (Cyquant assay).

Inhibition of Tumor Growth

Ability to inhibit angiogenesis-related diseases is evaluated by testing the ability of a peptide or an antibody to suppress tumor growth *in vivo*. In a primary tumor growth assay, a certain number of tumor cells such as B16 melanoma cells are injected subcutaneously in C57/J6 mice. The tumor cells are allowed to grow; treatment is initiated when the tumors become palpable. Tumor size is measured every day or every other day. The experiment is terminated at a pre-determined time point.

Administration

The compositions described previously may be administered by the topical, oral, rectal or parenteral (intravenous, subcutaneous or intramuscular) route. They may also be incorporated into biodegradable polymers for sustained release implanted at the disease site. The dosage of the compositions depends on the condition treated, the activity of the drug used, the route of administration, and other clinical factors such as severity of the disease and weight of the patient. The compositions are formulated in ways suitable for the specific route of administration. Formulations suitable for oral administration include capsules, cachets or tablets containing a predetermined amount of the active ingredient, powder or granules, solutions, suspensions, and emulsions. Formulations suitable for topical administration in the mouth include lozenges, pastilles, and mouthwashes. Formulations suitable for topical administration to the skin include ointments, creams, gels, pastes, and transdermal patches. Formulations for rectal administration may be presented as a suppository with a suitable base, while vaginal administrations may be presented as pessaries, tampons, creams, gels, pastes, foams, and sprays comprising the active ingredient in an appropriate carrier. Formulations suitable for parenteral

1 administration include aqueous and non-aqueous sterile injection solutions presented in
2 unit-dose or multi-dose containers. It should be also understood that, in addition to the
3 ingredients mentioned above, formulations of this invention might include other agents
4 conventional in the art having regard to the type of formulation in question.

5 The invention is further understood by the following non-limiting examples,
6 which are not to be construed in any way as imposing limitations upon the scope thereof.
7 On the contrary, it is to be clearly understood that resort may be had to various other
8 embodiments, modifications, and equivalents thereof which, after reading the description
9 herein, may suggest themselves to those skilled in the art without departing from the
10 spirit of the present invention and/or the scope of the appended claims.

11

12 **EXAMPLE 1**

13 *Effect of EP01 (SEQ ID NO: 1), EP02 (SEQ ID NO: 4), and EP03 (SEQ ID NO:*
14 *7) peptides on the bFGF-induced Proliferation of HUVECs.*

15 Proliferation assays familiar to those skilled in the art using human umbilical vein
16 endothelial cells (HUVECs) were employed in order to determine the effect of various
17 peptides and antibodies on the growth of bFGF-stimulated HUVECs.

18 Materials and Methods

19 The materials for this experiment included endothelial cells (HUVECs) and media
20 for their proliferation (Media 200, fetal bovine serum (FBS), gelatin, bFGF) (Paragon
21 Bioservices, Baltimore, MD), and Cell Titer 96 for detection of cell proliferation
22 (Paragon Bioservices, Baltimore, MD). Peptides EP01 (SEQ ID NO: 1), EP02 (SEQ ID

NO: 4), and EP03 (SEQ ID NO: 7) were synthesized by Multiple Peptide Systems (San Diego, CA).

HUVECs were routinely cultured to confluency in Media 200 containing 10% FBS. The cells were then trypsinized and plated in a 96-well plate pre-coated with 1% gelatin at 5000 cells per well per 100 μ L Media 200 containing 2% FBS. The cells were allowed to adhere for 24 hours. Subsequently, the media were aspirated and fresh Media 200 containing 0.5% FBS were added to the wells followed by the addition of various concentrations of peptides in the presence and absence of 20 ng/ml bFGF. The assay plates were incubated at 37°C, 5% CO₂ for 48 hours. At the end of the incubation period, cell proliferation was determined using cell counting (Cell Counter Model Z1, Coulter Incorporation, Miami, FL) or spectrophotometry. In the later case, the assay plates were incubated with Cell Titer 96 for 2 hours and the absorbance was recorded at 490 nm. The effect of the various peptides on the proliferation of endothelial cells was expressed as % inhibition. % Inhibition is defined by the following formula:

$$\frac{[\text{absorbance of cells treated with bFGF}] - [\text{absorbance of cells treated with bFGF and peptide}]}{[\text{absorbance of cells treated with bFGF}] - [\text{absorbance of untreated cells}]} \times 100 = \% \text{Inhibition of Proliferation}$$

Results

Peptides EP01 (SEQ ID NO: 1), EP02 (SEQ ID NO: 4), and EP03 (SEQ ID NO: 7) tested, here inhibited bFGF-induced HUVEC proliferation. The relative antiproliferative effects of EP01 (SEQ ID NO: 1), EP02 (SEQ ID NO: 4), and EP03 (SEQ ID NO: 7) are shown graphically in FIG. 2. For each point of FIG. 2, the error is

1 less than 10%. % Inhibition is defined in Materials and Methods. The IC₅₀ values of the
 2 antiproliferative effect of the peptides are reported below:

3

Peptide	IC ₅₀ of Antiproliferative Effect
EP01 (SEQ ID NO: 1)	210 μ M
EP02 (SEQ ID NO: 4)	235 μ M
EP03 (SEQ ID NO: 7)	245 μ M

4

5 EXAMPLE 2

6 *Production of murine polyclonal antisera that bind EP02 (SEQ ID NO: 4) and*
 7 *EP03 (SEQ ID NO: 7).*

8 Antibody production protocols familiar to those skilled in the art were employed
 9 in order to produce murine polyclonal sera, which recognize and bind to peptides with
 10 specific amino acid sequences.

11 Materials and Methods

12 Peptides EP02 (SEQ ID NO: 4) and EP03 (SEQ ID NO: 7) were conjugated with
 13 KLH, a highly immunogenic copper-containing protein, using a commercially available
 14 kit (Pierce, product number 77622). The resulting conjugated peptides were used for
 15 immunization of mice. After two booster immunizations, the mice were bled and murine
 16 anti-EP02 and anti-EP03 antisera were obtained. Various dilutions of these antisera were
 17 tested for their ability to bind 96-well plates coated with 2 μ g/ml EP02 and EP03.
 18 Specifically, 96-well plates were incubated for 2 hrs at room temperature with 50 μ l per
 19 well of either 2 μ g/ml EP02 or 2 μ g/ml EP03 in 50 mM Carbonate-Bicarbonate buffer,

pH 9.6 (Sigma, St. Louis, MO). Subsequently, the wells were emptied and non-specific binding was blocked with 200 µl of 3% non-fat dry milk in PBS (BioWhittaker, MD) (30 minutes, room temperature). The wells were washed three times with 300 µl PBS containing 0.1% Tween-20. A volume of 50 µl of polyclonal antisera diluted in PBS-0.1% Tween-20 was then added to the wells. After a 60 min incubation at room temperature, the wells were emptied and washed. This was followed by the addition of 50 µl of secondary antibody (goat anti-mouse IgG and IgM peroxidase-labeled abs) diluted in 200 µl PBS containing 0.1% Tween-20. After a 30-min incubation at room temperature, the wells were washed and 50 µl of a peroxidase substrate (ABTS, Kirkegaard and Perry) were added. Binding was measured at 405 nm.

Results

Both peptides were shown to be highly immunogenic as shown in FIG. 3. There was no cross-reactivity between the different antisera.

EXAMPLE 3

Production of monoclonal antibodies, which recognize and bind EP02 (SEQ ID NO: 4) and EP03 (SEQ ID NO: 7).

Monoclonal antibody production protocols familiar to those skilled in the art were employed in order to produce monoclonal abs, which recognize and bind to peptides with specific amino acid sequences

Materials and Methods.

Monoclonal antibodies (B2G4 and D2G11), which recognize and bind EP02 (SEQ ID NO: 4) and EP03 (SEQ ID NO: 7) respectively, were generated from previously produced antisera according to well-known methods of antibody production (Seon *et al.* Monoclonal antibody that defines a unique human T-cell leukemia antigen, *Proc. Natl. Acad. USA*, **80**, 845-849 (1983)). B2G4 and D2G11 mabs specifically recognized EP02 (SEQ ID NO: 4) and EP03 (SEQ ID NO: 7), respectively. These abs were also able to bind to the cell surface of HUVECs, as measured by a cell-based binding assay. Specifically, HUVECs were plated at 75% confluency in 96-well plates and stimulated with 2 ng/ml bFGF. After overnight incubation, the wells were emptied and washed with cold PBS. This was followed by addition of 200 µl of the binding buffer (10 mM MOPS pH 6.7 containing 250 mM sucrose and 0.4 mM ATP). The cells were then incubated with 20 µg of ab for 2 hrs at 37 °C. Subsequently, the wells were emptied and washed. After incubation with a fluorescein-labeled secondary ab for 30 min at 37 °C, specific binding is measured with a fluorometer.

Results

Monoclonal abs that recognize and bind EP02 (SEQ ID NO: 4) and EP03 (SEQ ID NO: 7) bind to the cell surface of proliferating HUVECs, as depicted in FIG. 4.

EXAMPLE 4

Monoclonal abs B2G4 and D2G11 inhibit bFGF-induced proliferation of HUVECs.

1 Materials and Methods

2 HUVEC proliferation assays in the presence of abs were performed as previously
3 described.

4 Results.

5 B2G4 and D2G11 mabs induce significant inhibition of bFGF-induced
6 proliferation of HUVECs, as depicted in FIG. 5.

8 **EXAMPLE 5**

9 *Isolation and sequencing of DNA encoding the V_H and V_L domains of B2G4.*

10 Materials and Methods

11 Approximately 1×10^7 hybridoma cells producing B2G4 antibody were grown in
12 T-75 flasks and then harvested for RNA isolation using S.N.A.P.TM Total RNA Isolation
13 Kit from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. cDNA
14 was synthesized using reverse transcriptase (SuperScriptTM III One-Step RT-PCR System
15 with Platinum® *Taq* DNA Polymerase kit from Invitrogen). PCR amplification was
16 performed in a 100 μ L reaction volume using 2 μ L cDNA, 1 μ L 10 mM dNTPs, 10 μ L
17 *Taq* polymerase buffer, 2.5 U *Taq* polymerase (from Promega, Madison, WI), and 20
18 pmol 5' or 3' primers in H₂O. Specific primers used herein were: heavy chain forward, a
19 mixture of 5'-ctt ccg gaa ttc SAR GTN MAG CTG SAG SAG TC-3' (SEQ ID NO:10),
20 5'-ctt ccg gaa ttc SAR GTN MAG CTG SAG SAG TCW GG-3' (SEQ ID NO:11), 5'-cct
21 ccg gaa ttc CAG GTT ACT CTG AAA GWG TST G-3' (SEQ ID NO:12), 5'-ctt ccg gaa
22 ttc GAG GTC CAR CTG CAA CAR TC-3' (SEQ ID NO:13), 5'-ctt ccg gaa ttc CAG
23 GTC CAA CTV CAG CAR CC-3' (SEQ ID NO:14), 5'-ctt ccg gaa ttc GAG GTG AAS

1 STG GTG GAA TC-3' (SEQ ID NO:15), 5'- ctt ccg gaa ttc GAT GTG AAC TTG GAA
 2 GTG TC-3' (SEQ ID NO:16), heavy chain reverse, 5'-gga aga tct GAC ATT TGG GAA
 3 GGA CTG ACT CTC-3 (SEQ ID NO:17), light chain forward, 5'-gg gag ctc GAY ATT
 4 GTG MTS ACM CAR WCT MCA-3' (SEQ ID NO:18), and light chain reverse, 5'-ggt
 5 gca tgc GGA TAC AGT TGG TGC AGC ATC-3' (SEQ ID NO:19) (Wang *et al.*
 6 Universal PCR amplification of mouse immunoglobulin gene variable regions: the design
 7 of degenerate primers and an assessment of the effect of DNA polymerase 3' to 5'
 8 exonuclease activity, *J. Immunol Methods*, **233**, 167-177 (2000)). The underlined letters
 9 in the primer sequences represent cloning sites, EcoRI (gaattc), BglII (agatct), SacI
 10 (gagctc), and SphI (gcatgc). For both heavy and light chains, forward primers are
 11 degenerates with S = C or G, R = G or A, N = A, C, G or T, M = A or C, W = A or T,
 12 V = A, C or G, and Y = C or T. Separate reactions were set up for heavy and light
 13 chains. Cycling conditions were: 94°C for 3 min, 30 cycles of a three-step program
 14 (94°C, 1 min; 45°C, 1 min; and 72°C, 2 min), 72°C for 10 min, and then cooled to 4°C
 15 (Perkin Elmer 9700). Amplified fragments were digested and separated on a 1% TAE
 16 gel. DNA was recovered from the agarose slices using a GeneClean II kit (Qbiogene,
 17 Carlsbad, CA). V_H and V_L fragments were cloned into the pUC19 vector and expressed
 18 in DH10B™ *E. coli* cells (Invitrogen). Plasmid DNA was isolated using the SNAP
 19 MiniPrep kit and sequenced by Retrogen (San Diego, CA).

20 Results

21 The results are shown in FIG. 6 and FIG 7. DNA sequence for V_H and V_L
 22 fragments are:

23 V_H sequence from EcoRI to BglII (SEQ ID NO: 8):

1 gaattcGAGGTGAASGTGGTGGGAATCTGGGGGAGGCTTAGTGAAGCCTGG
 2 AGGGTCCCTGAAACTCTCCTGTGCAGCCTCTGGATTCACTTTCAGTAGCTATG
 3 CCATGTCTTGGGTTCGCCAGACTCCAGAGAAGAGGCTGGAGTGGGTTCGCATC
 4 CATTAGTAGTGGTGGTAGCACCTACTATCCAGACAGTGTGAAGGGCCGATTC
 5 ACCATCTCCAGAGATAATGCCAGGAACATCCTGTACCTGCAAATGAGCAGTC
 6 TGAGGTCTGAGGACACGGCCATGTATTACTGTGCAAGAGGCCTACCATTTCG
 7 TTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGAGAGTCAGTCCTTCC
 8 CAAATGTCagatct

9 V_L sequence from SacI to SphI (SEQ ID NO: 9):

10 gagctcGATATTGTGATgACaCAatCTACAGCTTCCTTAGCTGTATCTCTGG
 11 GGCAGAGGGCCACCATCTCATGCAGGGCCAGCCAAAGTGTCAGTACATCTAG
 12 CTATAGTTATATGCACTGGTACCAACAGAAACCAGGACAGCCACCCAAACTC
 13 CTCATCAAGTATGCATCCAACCTAGAATCTGGGGTCCCTGCCAGGTTCAGTGG
 14 CAGTGGGTCTGGGACAGACTTCACCCTCAACATCCATCCTGTGGAGGAGGAG
 15 GATACTGCAACATATTACTGTCAGCACAGTTGGGAGATTccGCTCaCGTTCGGT
 16 GCTGGGACCAAGCTGGAGCTGAAACGGGCTGATGCTGCACCAACTGTATCCgc
 17 atgc

18 Comparison of the amino acid sequences encoded by SEQ ID NO: 8 and SEQ ID
 19 NO: 9 with the amino acid sequences of V_H and V_L domains of known murine antibodies
 20 (Carter et al. Humanization of an anti-p185HER2 antibody for human cancer therapy,
 21 *Proc Natl. Acad. Sci. USA* **89**, 4285-4289 (1992)) suggests that the highlighted areas of
 22 FIG. 6 and 7 represent potential CDR domains for the heavy and light chains of B2G4
 23 antibody.